Indoor Air 2013; 23: 142–147
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INDOOR AIR
doi:10.1111/j.1600-0668.2012.00799.x

Airborne fungal cell fragments in homes in relation to total fungal biomass

Abstract Fungal exposure may induce respiratory symptoms. The causative agents are compounds in the fungal cell wall. Fragments of microbes may be present in air samples but are not measurable using conventional spore counting or by the determination of viable organisms. This study assesses the proportion of fungal cell biomass and endotoxin in different particle size fractions in air samples from homes. Air samples were collected from 15 homes using a cyclone sampler, collecting particles in three aerodynamic size fractions: <1.0, 1.0–1.8, and >1.8 μm. N-Acetylhexosaminidase (NAHA) was determined as a marker of fungal cell biomass. Endotoxin was determined using the *Limulus* amebocyte lysate method. NAHA and endotoxin in the size range <1.0 μm comprised up to 63% (mean 22.7%) and 96.3% (mean 22.6%) of the total concentrations, respectively. There were significant relationships between the amounts of NAHA and endotoxin in the total amount and in the size fraction >1.8 μm but not in the smaller fractions. The results demonstrate significant amounts of fungal cell biomass and endotoxin in particles <1.0 μm. Homes with reported mold damage had a lower concentration of NAHA in particles <1.0 μm than homes without mold damage. To assess airborne exposure for diagnostic and preventive purposes, measurement techniques that include this fraction should be considered.

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Key words: Fungi; Endotoxin; β-Glucan; Particle fractions; N-Acetylhexosaminidase; Air sampling.

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Received for review 5 May 2012. Accepted for publication 25 June 2012.

Practical Implications

Considerable amounts of biologically active microbial agents may be present in airborne fractions of microbial cells smaller than 1.0 μm. For optimal health risk assessment of indoor airborne microbes, sampling techniques that collect the small fraction should be employed.

Introduction

Domestic exposure to fungi comprises a risk of symptoms from the airways, eye irritation, and unusual fatigue (Bush et al., 2006; Fisk et al., 2007; Kaufman et al., 1995; Zock et al., 2002). Initially, it was believed that these adverse reactions were attributable to fungal antigen–induced IgE antibodies, which caused clinical symptoms through effector cells such as mast cells. It is now known that the asthmatic inflammation may be brought about by a variety of mechanisms involving the innate as well as the adaptive immune systems (Fajt and Wenzel, 2009; Holt and Sly, 2009). It is also known that a number of agents in the fungal cell wall, such as β-glucan, chitin, and peptides, may induce an inflammatory response through effects on the innate immune system. Beta glucan may induce both Th1- and Th2-derived reactions (Rylander, 2010), and chitin induces the secretion of different inflammatory mediators (Young et al., 2007).

During growth and atmospheric desiccation, fungi may produce non-viable small particles that contain bioactive agents in the fungal cell wall (Cho et al., 2005; Górny et al., 2002; Madsen et al., 2009; Reponen et al., 2007). Furthermore, fungal or bacterial DNA may be present in airborne particles (Despres et al., 2007). According to one study, the release of such fungal fragments is dependent on fungal species, the air velocity, and the texture and vibrations of the contaminated material (Górny et al., 2002). There was no correlation between the number of spores and the amount of fragments. In a study on fungal and bacterial aerosols in biofuel plants, the mass concentration of small particles (PM_1) was associated with the amount of β-glucan and the enzyme N-acetylhexosaminidase (NAHA), a measure of total fungal cell biomass (Madsen et al., 2009).

Previous measurements of fragments in moldy houses, using β-glucan as a fungal cell marker, have shown that the ratio of β-glucan mass in the fragment size fraction (<1 μm) to that in the spore size fraction (>2.25 μm) can be up to 2.2 (Reponen et al., 2007). In an experiment where different species of fungi were aerosolized, it was found that *Stachybotrys chartarum* released proportionally more fragments than *Aspergillus versicolor* (Cho et al., 2005). For *S. chartarum*, the deposition ratio in the lower airways of an infant was calculated to be 4–5 times higher than that for an adult. One study demonstrated that small fragments comprised 6.3% of the total number of fungal particles (Li and Kendrick, 1995).

The fragments of fungal cells may remain airborne for longer time than larger particles including fungal spores. As they have a higher penetration into the airways, they probably contribute significantly to the risk of symptoms, as shown in a study on asthma severity and respiratory function (Delfino et al., 1997).

In view of the above, determination of fungal cell mass could be a more precise measure of the potential exposure than counts of viable colonies or spores. This study was undertaken to determine the proportion of airborne fungal cell fragments in homes. In view of the relationship between respiratory symptoms and endotoxin shown in previous studies (reviewed in Rylander, 2007), the amount of bacterial endotoxin was also determined.

Material and methods

Fifteen homes were chosen from the study cohort of Cincinnati Childhood Allergy and Air Pollution Study in the Greater Cincinnati and northern Kentucky area (Reponen et al., 2011). The size of the homes varied from 770 to 3200 square feet, and mold damage was reported in nine of the homes (Table 1). The temperature and humidity (mean ± s.d.) in the homes were 22.8 ± 2.4°C and 37.1 ± 10.4%, respectively. The number of occupants (mean ± s.d.) in the homes was 4 ± 2. Pets were reported in eight homes.

Air samples were collected from the main activity room of the children (either a living room or a bedroom) for 24 h using a NIOSH-developed two-stage cyclone sampler. The sampler consists of two screw-top 1.5-ml microcentrifuge tubes (Model No. 506-624: PGC Scientifics Corp., Frederick, MD, USA) and a 37-mm filter holder with 0.8-μm polycarbonate filter (SKC Inc., Eighty Four, PA, USA). The minimum efficiency of the collection filters was found to be 92% for 90-nm particles (Singh et al., 2011). At an airflow rate of 3.5 l/min, the 50% cut-off diameters of the first and second tubes are 1.8 and 1.0 μm, respectively. Thus, the sampler collects aerosolized biocontaminants into three particle size ranges: <1.0, 1.0–1.8, and >1.8 μm. The design and performance characteristics of the NIOSH two-stage cyclone sampler have been described previously (Lindsley et al., 2006). Two samplers were operated side-by-side. One was used for NAHA analysis, and the other one was used for endotoxin analysis.

Fungal cell biomass in the different fractions was determined by measuring the activity of N-acetylhexosaminidase (NAHA) (Rylander et al., 2010). For the analysis, one mL of a fluorogenic enzyme substrate (4-methylumbelliferyl *N*-acetyl-beta-D-glucosaminide, Mycometer A/S, Copenhagen, Denmark) was added to the filter. After incubation, usually 30 min – depending on the room temperature, 2 ml of an alkaline developer was added. The liquid (3 ml) was placed in a

Table 1 Characteristics of the 15 study homes

Home ID	Average temperature (°C)	Average relative humidity (%)	Water damage[a]	Mold damage[a]	Number of occupants	Pets	Area of home (sq. feet)
1	25.0	29.0	NO	YES	4	2 dogs	2800
2	21.8	25.5	YES	YES	5	2 cats	2251
3	21.8	57.2	YES	YES	4	1 dog	1021
4	22.8	41.0	NO	YES	2	0	700
5	18.6	26.0	NO	YES	3	0	1484
6	24.7	44.0	NO	NO	3	0	773
7	21.5	53.5	YES	NO	3	2 dogs	1404
8	20.1	32.0	NO	NO	4	0	2662
9	24.9	29.0	NO	NO	5	0	2453
10	23.0	43.0	NO	NO	5	1 cat, 1 dog	2128
11	18.9	30.0	YES	YES	4	0	3200
12	25.9	35.0	YES	YES	3	1 cat, 1 dog	1271
13	22.2	24.0	YES	NO	4	2 cat, 5 dog	1924
14	25.0	41.0	YES	YES	4	2-dog	1786
15	25.3	47.0	YES	YES	9	0	1762

[a]Reported by the occupants.

cuvette, and the fluorescence values were read in a fluorimeter (Picofluor; Turner Designs, Sunnyvale, CA, USA). One count is equal to 2.3 ng *Aspergillus oryzae* NAHA. To avoid random scatter, the counts read in the fluorimeter were divided by 10 and given as a round figure. This was divided by the air sampling volume and expressed as units/m^3 (NAHA U/m^3). The detection limit is 0.019 NAHA U/m^3 for the <1.0-μm fraction and 0.005 U/m^3 for the intermediate and coarse particle size fractions.

Endotoxin in the different fractions was measured using the *Limulus* amebocyte lysate method (Pyrochrome® Endotoxin Detection Reagent Kit; Associates of Cape Cod, Inc., East Falmouth, MA, USA) according to the manufacturer's instructions. The endotoxin data are presented as endotoxin units/m^3 (EU/m^3). The limit of detection for endotoxin was 0.052 EU/m^3 for the <1.0-μm fraction and 0.014 EU/m^3 for the intermediate and coarse particle size fractions.

To investigate the potential bounce of fungal spores from the cyclone tubes of the two-stage cyclone sampler, spore counting was made in sample extracts of the first two homes. Extracts were filtered through a mixed cellulose esterase filter, dried, cleared with acetone vapor, stained with lactophenol cotton blue, and identified under the microscope as described previously (Adhikari et al., 2003).

Statistical analysis

Values below the limit of detection were divided by two before data analysis. Correlation coefficients were calculated between different size-selective data sets for both NAHA and endotoxin. The data were statistically analyzed by analysis of variance (ANOVA) and *t*-test. Scheffe's test was used to locate the difference that ANOVA identified. All statistical tests were performed using the SPSS Statistics 17.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

The average amounts of NAHA and endotoxin in all samples are reported in Table 2.

The amounts of NAHA and endotoxin were higher in the <1.0-μm particle size fraction than in the 1.0- to 1.8-μm fraction. According to ANOVA, the values for NAHA < 1.0 μm, NAHA > 1.8 μm, and NAHA 1–1.8 μm were significantly different from each other ($P < 0.001$). The post hoc Scheffe test data showed that NAHA < 1.0 μm was significantly different from NAHA > 1.8 μm ($P = 0.004$), but not from NAHA 1–1.8 μm. NAHA > 1.8 μm was significantly different from other two groups ($P = 0.004$ and <0.0001, respectively), and NAHA 1–1.8 μm was significantly different from NAHA > 1.8 μm ($P < 0.0001$).

Endotoxin <1.0 μm, endotoxin > 1.8 μm, and endotoxin 1–1.8 μm were significantly different from each other ($P < 0.01$). The post hoc Scheffe test data for endotoxin showed results similar to those for NAHA: endotoxin <1.0 μm was significantly different from endotoxin > 1.8 μm ($P = 0.021$), but not from endotoxin 1–1.8 μm. Endotoxin >1.8 μm was significantly different from the other two groups ($P = 0.021$ and 0.003, respectively), and endotoxin 1–1.8 μm was significantly different from endotoxin >1.8 μm ($P = 0.003$).

The activity of NAHA in the particle size <1.0 μm ranged from 0% to 62.5% of the total activity [mean 22.7%, standard error of the mean (s.e.m.) 5.5] and that of endotoxin from 0.4% to 96.3% (mean 22.6%, s.e.m. 8.4). There were significant relationships between NAHA and endotoxin for the total activity ($P < 0.003$) and for size >1.8 μm ($P = 0.001$), but not for sizes 1.0–1.8 and <1.0 μm. The distribution of the proportion of NAHA and endotoxin in the <1.0-μm fraction in relation to the total amount is shown in Figure 1.

The number of samples with a higher proportion of the <1.0-μm fraction was larger for NAHA than for endotoxin. Most NAHA ratios were closer to the 1:1 line than the endotoxin ratios. Furthermore, most endotoxin samples had <20% of the total activity in the size fraction <1.0 μm. Figure 2 shows the relationship between the total amount and the <1.0-μm fraction for NAHA.

Table 2 Mean (standard error of mean) amounts of N-acetylhexosaminidase (NAHA) and endotoxin in different particle size fractions

Cell wall component	Amount in different particle size fractions (μm) <1.0	1.0–1.8	>1.8	Total amount
NAHA (U/m^3)	1.08 (0.30)	0.24 (0.09)	3.56 (0.78)	4.87 (0.87)
Endotoxin (EU/m^3)	0.36 (0.18)	0.10 (0.04)	1.25 (0.37)	1.71 (0.50)

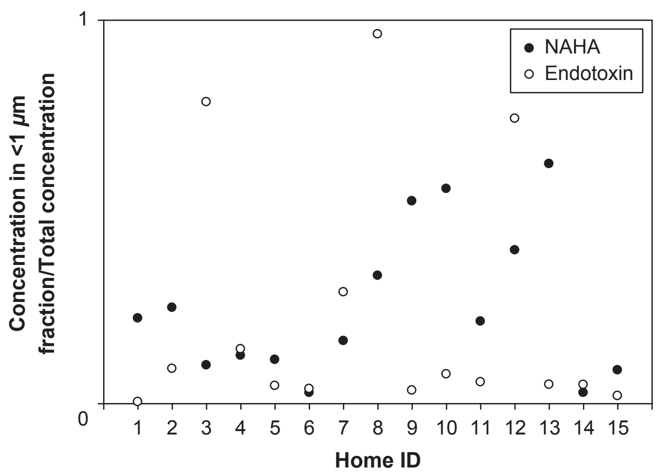

Fig. 1 The proportion of NAHA and endotoxin in the <1.0-μm particle size fraction in relation to the total amount

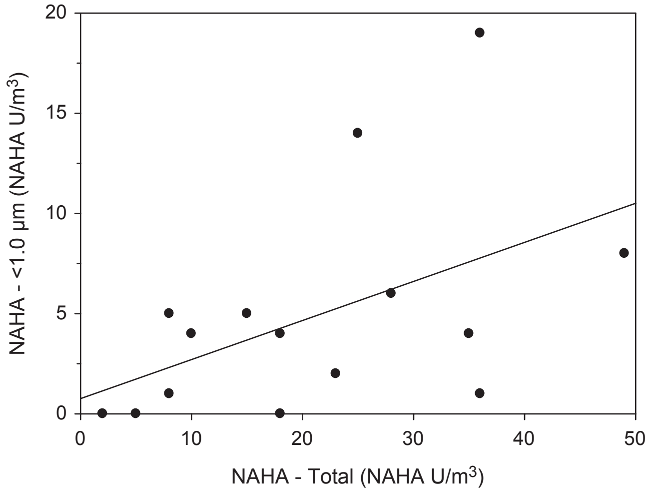

Fig. 2 Relationship between total amount and <1.0-μm fraction for NAHA (r = 0.545, P = 0.036)

The relationship between the total concentration and the <1.0-μm fraction was statistically significant. There was no significant relationship for endotoxin (r = 0.350, P = 0.201).

In houses with reported mold problems, the amount of NAHA in particles <1.0 μm was lower than that in houses with no mold (Table 3). This difference became even stronger when one outlier was removed. Similar, but not significant, trend was seen for endotoxin.

Fungal spores were counted and identified in size-selective samples collected from two homes. This analysis showed that the proportion of spores in the size fraction of 1.0–1.8 μm was 11.5% and 10.9% for the two homes, respectively, compared with the total number of spores in each home, respectively. The corresponding proportions of spores in the <1.0-μm size fraction were 1.2% and 2.1%. The >1.8-μm fractions contained primarily spores of *Aspergillus, Penicillium, Cladosporium*, ascospores, and basidiospores, whereas the <1.0-μm fractions contained only *Aspergillus* and *Penicillium*.

Discussion

The main result from the study was that the small particle size fractions of fungi and endotoxin may comprise a significant part of the total amount, that the concentration of NAHA in the <1.0-μm size fraction was lower in moldy homes, and that airborne NAHA and endotoxin correlated strongly in the large particle size fraction (>1.8 μm), but not in the smallest fraction.

Table 3 The amounts of N-acetylhexosaminidase (NAHA) and endotoxin in the <1.0-μm size fraction in homes with and without mold problems mean amount (s.e.m.)

Agent	Homes without mold problems (N = 6)	Homes with mold problems (N = 9)	P-value (Mann–Whitney test)
NAHA (U/m^3) in <1.0 μm fraction	1.9 (0.6)	0.5 (0.1)	0.036
One outlier excluded	2.3 (0.6)	0.5 (0.1)	0.002
Endotoxin (EU/m^3) in <1.0-μm fraction	0.7 (0.4)	0.1 (0.1)	NS

NS, not significant.

The study has some limitations. First, the sample size was relatively small. Second, the aerodynamic size-selective fractionation of particles by the two-stage cyclone sampler may not be highly precise as larger spores may bounce in the cyclone tubes and may contaminate the submicron fraction. The results from the spore counting in the two samplers suggest, however, that the proportion of spores reaching the last collection stage (<1.0 μm) was low, and thus, spore bounce appears to be negligible.

Fungal cell biomass was determined by measuring NAHA. Although this enzyme is not specific for fungi, several studies in complex environments have demonstrated a good correlation with fungal biomass (Madsen, 2003; Miller et al., 1998). Significant correlations with total spore counts were found in air samples and in dust generated from biomass in a biofuel plant (Madsen et al., 2009). Strong correlations were found between fungal biomass (gravimetric weight) and NAHA in fungal species grown on nutrient agar and between ergosterol and NAHA activity on gypsum boards (Reeslev et al., 2003). In field studies, measurements of NAHA were found to identify buildings with mold problems with a high specificity and sensitivity (Rylander et al., 2010). High levels of NAHA exposure have been found in homes of patients with sarcoidosis and with nocturnal asthma (Terčelj et al., 2011, 2012).

The results confirm findings from previous studies in that fungal and bacterial fragments may comprise a substantial part of the airborne exposure to microorganisms in homes (Kujundzic et al., 2006; Reponen et al., 2007). This could explain why poor relationships between symptoms and microorganisms, measured using conventional techniques such as spore counting, have been found in some previous studies (Holme et al., 2010).

There was a significant relationship between NAHA and endotoxin in the particle size >1.8 μm, but not in the smaller fractions. Also, the proportion of the <1.0-μm fraction in relation to the total amount was higher for NAHA than for endotoxin and was related to the presence of mold damage. This suggests that the formation of respirable and submicron cell fractions in indoor environmental conditions differs between fungi and bacteria and also implies that dry conditions favor the creation of smaller fungal particles. This has also been shown in a study where the aerosolization of fungal spores was studied at different levels of humidity (Madsen, 2012). At a high relative humidity, about 30% of the particles were of respirable size as compared to 70% at a low humidity.

The results from this and previous studies suggest that methods that collect and measure fungal and bacterial fragments should be used for exposure determination and risk assessment of fungal and endotoxin exposure in homes. For the determination of fungal biomass, determinations of NAHA as used here or β-glucan as used in several previous studies are suitable. Regarding β-glucan, the two commonly used methods for analysis are the *Limulus* amebocyte lysate test and an immunological assay (ELISA) (e.g., Douwes et al., 1996; Rylander, 1997). The two methods have been compared in an extensive study, evaluating the accuracy and specificity for the detection of eight standard preparations of β-glucan, the sensitivity for the analysis of specific samples of fungi, and the content of β-glucan in indoor dust samples (Iossifova et al., 2008). There was no correlation between the methods in floor dust samples, but a weak correlation if the amount of β-glucan was expressed per surface area. The Limulus test had a higher sensitivity (1000-fold) and specificity and was more accurate in measuring the concentrations of β-glucan standards. The sensitivity of the ELISA method is too low to allow for the analysis of airborne samples. These results demonstrate that a *Limulus* amebocyte lysate–based test is more appropriate to estimate the airborne exposure to fungi.

In conclusion, the determination of different particle size fractions demonstrated that both fungal biomass and endotoxin in small particles may comprise a significant portion of the total exposure. Sampling techniques that include the small fractions are thus required for correct respiratory health risk assessments of indoor microbial exposure. The fragmentation processes in fungi and Gram-negative bacteria are likely to be different.

Acknowledgements

This study was partially supported by the Grant No. OHLHH0199-09 from the Healthy Homes Technical Studies Program of the US Department of Housing and Urban Development. The authors thank Drs. William G. Lindsley and Bean T. Chen at NIOSH for providing the NIOSH two-stage cyclones for the study. The assistance of Ms. Olivia Thomas, Mrs. Reshmi Indugula, and Mr. Christopher Schaffer for the collection of air samples from homes, processing of samples, and laboratory work is gratefully acknowledged.

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